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Rapid diagnosis of smear-negative pulmonary tuberculosis via fibreoptic bronchoscopy: utility of polymerase chain reaction in bronchial aspirates as an adjunct to transbronchial biopsies

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Fibreoptic bronchoscopy was performed on 190 patients with chest radiographic lesions and negative sputum smears for acid-fast bacilli. Aside from obtaining transbronchial biopsies for histological examination, bronchial aspirate specimens were also tested for *Mycobacterium tuberculosis* complex DNA by a conventional polymerase chain reaction (PCR) technique. Of 177 transbronchial biopsies performed, a diagnosis was found in 64 cases [43 cases of tuberculosis (TB), 17 cases of lung carcinoma and four cases of other infective/inflammatory diseases] giving a diagnostic yield of 36·2%. PCR was positive in 105 of 108 finally diagnosed cases of TB and 22 of 82 non-TB cases. The sensitivity, specificity, positive predictive value and negative predictive value of PCR when applied to bronchial aspirate specimens for diagnosing smear-negative pulmonary TB were 97·2%, 73·2%, 82·7% and 95·2% respectively. Therefore, detection of *M. tuberculosis* complex DNA in bronchial aspirates by PCR might have an adjunctive place to transbronchial biopsies in the rapid diagnosis of smear-negative pulmonary tuberculosis.

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Introduction

Pulmonary tuberculosis (TB) is an important respiratory infection and health problem worldwide. Early diagnosis and treatment are important components in the control of this infectious disease. Direct smear examination of sputum is a simple and rapid diagnostic method but is limited by low sensitivity. Therefore, other methods of rapid diagnosis of smear-negative TB have become areas of intense research in recent years. Fibreoptic bronchoscopy has been reported to be useful in the early diagnosis of TB by histological examination of transbronchial biopsies (1–3). In recent years, a new diagnostic technique by the detection of DNA of *Mycobacterium tuberculosis* complex after amplification by polymerase chain reaction (PCR) has been widely reported (4–11).

In this study, we investigated the application of PCR in bronchial aspirate specimens as an adjunct to transbronchial biopsies in the rapid diagnosis of smear-negative TB.

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Patients and Methods

One hundred and ninety patients with or without respiratory symptoms were enrolled into this study. They all had lesions on chest radiographs, for which pulmonary TB was a differential diagnosis. All patients also had sputum negative for acid-fast bacilli by direct microscopy on at least two occasions. Fibreoptic bronchoscopy was performed on these patients. In addition to obtaining transbronchial biopsies for histological examinations and saving bronchial aspirates for cytological examinations and mycobacterial culture, about 5 ml of bronchial aspirates were saved and sent for PCR study. The results of the PCR study were correlated retrospectively with the final diagnoses reached.

FIBREOPTIC BRONCHOSCOPY PROCEDURE

The investigations were performed by experienced physicians. Bronchoscopy was done by a transoral or transnasal route using a Pentax SB15X fibreoptic bronchoscope. Patients were premedicated with atropine 0·3 to 0·6 mg and pethidine 30 to 50 mg i.m. Lignocaine hydrochloride was used for topical anaesthesia of the larynx and tracheobronchial tree. After examination of these structures, normal saline was instilled into the orifices of the bronchopulmonary segments at which the lesion was likely to be located,

and was then aspirated back. Transbronchial lung biopsy was taken from the same bronchopulmonary segments.

MYCOBACTERIAL EXAMINATION METHOD OF THE BRONCHIAL ASPIRATE SPECIMENS

A modified Petroff method was used for the mycobacterial culture of the bronchial aspirate specimens. After sodium hydroxide decontamination and neutralization, the specimen was centrifuged at 3000 *g* for 20 min and two loopsful of the sediment was directly inoculated into the Löwenstein–Jensen medium. The medium was examined weekly for up to 12 weeks. Identification and drug susceptibility tests were done for positive cultures.

PREPARATION OF THE BRONCHIAL ASPIRATE SPECIMEN AND EXTRACTION OF DNA FOR PCR

Bronchial aspirate specimens (0·5 ml) were pretreated with 1·0 ml 4% (w/v) sodium hydroxide, shaken at 2500 rpm for 25 min, 1·5 ml penicillin water (0·2 IU ml^{-1}) was added, mixed and centrifuged at 5000 *g* for 20 min. The supernatant was discarded and the pellet was washed twice with 2·0 ml penicillin water, and then resuspended in 1·0 ml 1 × TE buffer. Lysozyme was then added to the suspension, mixed, and incubated at 37°C for 1 h. The solution was further mixed with 70 ml of sodium dodecyl sulphate and 6 μl of Proteinase K, incubated at 65°C for 10 min, before the addition of 100 ml of 5 M NaCl. Eighty μl of N-cetyl-N,N,N,-trimethyl ammonium bromide/NaCl mixture was then added and vigorously mixed with the mixture. DNA was then extracted from the milky mixture by equal volume of chloroform : isoamyl alcohol (24:1, v/v). After centrifugation, the supernatant was transferred to a fresh microcentrifuge tube. Isopropanol was then added to precipitate the DNA. After centrifugation, the DNA pellet was then washed with cold ethanol, repelleted by centrifugation and finally dissolved in distilled water until used.

POLYMERASE CHAIN REACTION/TECHNIQUE

A pair of 20-base synthetic oligonucleotides that flank a 245-base region of insertion sequence IS6110/986 for *M. tuberculosis* was synthesized. The sequences of the oligonucleotide primers were:

5′CGTGAGGGCATCGAGGTGGC3′ and
5′GCGTAGGCGTCGGTGACAAA3′

Another oligonucleotide pair of 20 bases in length located between the two primers was synthesized to be used as the hybridization probes and their sequences were:

5′GAACGGCTGATGACCAAACT3′ and
5′ACGTAGGCGAACCCTGCCCA3′

Both the oligonucleotide primers and probes were synthesized using an oligonucleotide synthesizer (Millipore) using the reagents supplied by the same company.

DNA amplification was performed in a final volume of 50 μl which contained 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin, 20 μl 1·25 mM (each) deoxynucleoside triphosphates, 1U of Taq polymerase, 10 μl of the extracted DNA and 200 ng of each oligonucleotide primers. The reaction was performed in an automated thermal cycler. The amplification procedure was initiated by denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 2 min, and finally elongation at 72°C for 4 min.

15 μl of the PCR products were examined under UV illumination in the form of electrophoretic spread in 0·8% agarose gel with ethidium bromide staining. The 245 bp band identified was separated by electrophoresis and processed by Southern blotting and hybridization with the labeled 20 bp oligonucleotide probes homologous to the middle portion of the insertion sequence IS6110/986. The hybridized products were viewed with a chemiluminescence system (ECL system) for identification of the *M. tuberculosis* DNA.

To avoid contamination, the DNA extraction, PCR, and electrophoresis procedures were performed in three separate laboratories. Reagents for every step were thoroughly checked and sterilized. Positive control using DNA extracted from MTB H37Rv and negative control without DNA were employed to monitor the PCR procedure.

DIAGNOSTIC CRITERIA OF TUBERCULOSIS IN THE PATIENTS STUDIED

Diagnosis of TB was based on histological, bacteriological and clinical grounds. Based on different evidences, TB patients can be divided into the following categories: those with:

(a) a positive transbronchial biopsy showing the presence of granulomatous inflammation with or without demonstrable acid-fast bacilli; and a positive culture of the bronchial aspirate and/or sputum for *M. tuberculosis*.
(b) only a positive transbronchial biopsy showing the presence of granulomatous inflammation with or without stainable acid-fast bacilli.
(c) only a positive culture of the bronchial aspirate and/or sputum for *M. tuberculosis*.
(d) none of the above, but with the diagnosis of TB confirmed by other investigative procedures such as needle biopsy or open biopsy of the lung.
(e) none of the above, but with the diagnosis of TB made on clinical and radiological grounds only.

Most importantly, patients in all groups showed improvement with anti-tuberculous drug treatment.

Results

Two hundred patients were enrolled into the study. Ten patients were lost to follow-up and the final diagnosis could

TABLE 1. Demographic data of 190 studied patients

Gender	no. (%)
Male	146 (76·8%)
Female	44 (23·2%)
Age	
Range	15–80 years
Mean	51·5 years
Ethnic group	no. (%)
Chinese	188 (99%)
Filipino	1 (0·5%)
Indian	1 (0·5%)

TABLE 2. Results of transbronchial biopsies

Diagnosis	Number
Tuberculosis	43
Carcinomas	17
Bronchiolitis obliterans and organizing pneumonia	1
Cryptococcosis	1
Actinomycosis	1
Aspergillosis	1
Non-diagnostic	113
Total	177

not be ascertained and they were thus excluded. The results of the remaining 190 cases were analysed. The demographic data of these patients are as shown in Table 1.

All patients underwent fibreoptic bronchoscopy. Transbronchial biopsies were not done in 13 cases because of severely compromised lung function. There was no mortality or significant morbidity associated with the procedure. Out of the 177 transbronchial biopsies, a conclusive diagnosis was obtained in 64 cases giving a diagnostic yield of 36·2% (Table 2).

Based on the diagnostic criteria alluded to earlier, a final diagnosis of TB was reached in 108 of the 190 cases studied. PCR was positive in 105 cases. In the remaining 82 non-tuberculous patients, PCR was positive in 22 cases (Table 3). The overall sensitivity, specificity, positive and negative predictive values of PCR in the diagnosis of TB were 97·2%, 73·2%, 82·7% and 95·2% respectively.

Among the 17 cases with transbronchial biopsies that yielded lung carcinoma, two of them subsequently had bronchial aspirates that yielded *M. tuberculosis* on culture. These latter patients had concomitant lung cancer and pulmonary TB. The bronchial aspirates for PCR were also positive in these two cases.

In the group of non-TB patients, 12 of 22 PCR positive cases had radiographic evidence of previous TB. This proportion seemed to be higher when compared with the PCR negative group (22 of 60) but the difference has not reached statistical significance (χ^2 test, $P=0·07$).

Discussion

The diagnosis of smear-negative TB is both a common and important clinical problem. In areas of high prevalence of TB like Hong Kong, TB is often the cause of persistent chest radiograph lesions. However, other conditions, lung carcinoma in particular, have to be considered as alternative diagnoses. Empiric TB treatment for radiographic lesions may carry a risk of delaying the diagnosis and administration of appropriate treatment for the non-TB disorders.

The usefulness of fibreoptic bronchoscopy in the diagnosis of sputum smear-negative TB has been well reported (1–3). It is a simple, safe and cost-effective procedure. A definitive diagnosis could be reached in over 90% of cases by combining histological and mycobacteriological study of specimens obtained (1,2). Transbronchial biopsy, apart from its usefulness in rapid diagnosis of TB, is also important in diagnosing other pulmonary diseases like lung carcinoma. In our study, the diagnostic yield of transbronchial biopsy in pulmonary tuberculosis was found to be 43/108 (39·8%) corroborating with findings of other series (1,2). On the other hand, examination for acid-fast bacilli by direct microscopy using bronchial aspirate and bronchial brush specimens has produced quite variable

TABLE 3. Results of PCR in bronchial aspirates in 190 patients studied

	TB					Non-TB cases
	[a]	[b]	[c]	[d]	[e]	
PCR (+)	31	10	31†	8	25	22 (12)*
PCR (−)	2	0	0	0	1	60 (22)*

[a] positive transbronchial biopsy+positive MTB culture.
[b] positive transbronchial biopsy only.
[c] positive MTB culture only.
[d] diagnosis made by other investigations.
[e] clinical and radiological diagnosis.
( )* number of patients with evidence of previous pulmonary tuberculosis.
†including two patients with concomitant TB and lung cancer.

yields (1–3). Such examinations were not investigated in this study.

PCR is a new molecular biology technique which has widespread clinical applications in medicine (4). The presence of an infective agent in the clinical specimens can be demonstrated by its DNA sequence after amplification. The insertion sequence IS6110/986 has been found to be specific for *M. tuberculosis* complex which allows differentiation from other non-tuberculous mycobacteria (5). Since the early 1990s, numerous studies on diagnosis of TB using PCR technique from various centres have been published. The reported sensitivity was in general very high. However, the sensitivity becomes much lower when only smear-negative cases are considered. Clarridge examined over 5000 respiratory specimens among which 218 were positive for *M. tuberculosis*. PCR was positive in 83·5% of these cases. However, it was positive in 62% (45 of 73) of smear-negative cases (8). Similar results were found in a study by Nolte in which the sensitivity of PCR in the diagnosis of tuberculosis was 95% (105 of 110) but in smear-negative cases, PCR was positive in only eight of 14 (57%) (9). In our study, the sensitivity of PCR in diagnosis of TB was found to be very high. Bronchial aspirates, the specimens examined, were taken directly from the broncho-pulmonary segments in which the lesions were located. This may result in a better yield than examining sputum specimens, as the latter are more liable to be confounded with sampling bias. The attendant low specificity of the PCR test in our patients studied is unsatisfactory and requires further investigation and explanation. We conjecture that the rather sensitive PCR technique might also amplify DNA from very small number of dormant tubercle bacilli from the inactive TB lesions and gave rise to false-positive results. Although this possible cause for false-positive PCR results has not been properly studied, it has also been noted in some other studies. For example, in a study by Schluger, PCR was positive in 12 of 17 patients with a prior history of tuberculosis but no active pulmonary TB (10). Similarly, Querol found false-positive PCR in 10 of 44 patients with old pulmonary TB (11). About the usefulness of PCR when applied by routine clinical practice, Grosset and Mouton opined in a journal's editorial that PCR by itself should not be used as a rapid diagnostic tool for TB because of insufficient reliability (12).

Another genuine limitation to the routine use of PCR in diagnosis of TB is its high cost. Although the cost of a PCR test ranges widely (from about $7 US for in-house PCR to about $30 US for PCR by commercial kits), this is certainly prohibitive for routine application of the technique for diagnosis of TB for developing countries.

Finally, the diagnosis of TB by PCR does not exclude the presence of another pathology, especially when the prevalence of both diseases are high. This has been alluded to under the Results section for the two cases of concomitant TB and lung carcinoma.

Conclusion

Our study has demonstrated again, as has been previously shown, that bronchoscopy with transbronchial biopsy is very useful in the rapid diagnosis of smear-negative TB. PCR, the new molecular biology technique which has been studied in recent years, also has a definite role in the rapid diagnosis of TB. It might have an adjunctive role to transbronchial biopsy in a clinical setting. We suggest bronchoscopy and transbronchial biopsies to be done in sputum smear-negative patients for rapid diagnosis of TB and to exclude other pathology for undiagnosed lung lesions. In those cases when transbronchial biopsies are non-diagnostic or cannot be done because of severely compromised lung function, a PCR test of the bronchial aspirate specimens collected could be a useful adjunct in the early diagnosis of TB.

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